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(54) Title: GROWTH FACTOR SPLICE VARIANT

(57) Abstract: The invention relates to a polynucleotide sequence encoding a naturally occurring splice variant of human betacellulin (BTC), designated BTC-β. The polynucleotide sequence of the BTC-β lacks the sequence encoding the last C_5-C_6 disulphide loop of the epidermal growth factor $CX_7CX_4C_{10}CX_1CX_8C$ motif, which is normally present in the gene encoding the authentic BTC. The BTC-ß may be used for treating conditions mediated or modulated by ErbB receptors. The invention also provides methods for producing the BTC-β by recombinant DNA techniques and antibodies against the BTC-β.

GROWTH FACTOR SPLICE VARIANT

This invention relates to a polynucleotide sequence which encodes a naturally occurring splice variant of human betacellulin (BTC), designated BTC-β, which lacks polynucleotide sequences normally present in the gene which codes for authentic BTC. The betacellulin variant of the invention may be used as a pharmaceutical for the treatment of certain human diseases. The invention also provides methods for producing BTC-β by recombinant DNA techniques.

BACKGROUND OF THE INVENTION

All references, including any patents or patent applications, cited in this specification are hereby incorporated by reference. No admission is made that any reference constitutes prior art. The discussion of the references states what their authors assert, and the applicants reserve the right to challenge the accuracy and pertinency of the cited documents. It will be clearly understood that, although a number of prior art publications are referred to herein, this reference does not constitute an admission that any of these documents forms part of the common general knowledge in the art, in Australia or in any other country.

The cellular growth and differentiation of multicellular organisms is initiated and regulated by intercellular interactions mediated by a multiplicity of peptide growth factors. Many of these peptide growth factors are implicated in a wide variety of physiological and pathological processes, including signal transduction, cell survival, differentiation, cell adhesion, cell migration, immune response, hematopoiesis, inflammation, tissue repair, atherosclerosis and cancer. Consequently there is a great deal of interest in isolating, characterising and defining the functional mechanisms of peptide growth factors, not only in order to understand their role in the basic mechanisms underlying normal mammalian growth and

development, but also because of their potential therapeutic use.

In almost all the processes described above, peptide growth factors exert their biological effects by interacting with the extracellular domain(s) of transmembrane receptor tyrosine kinases (RTKs). Most RTKs belong to small groups of highly homologous receptors which bind similar ligands and maintain inter-receptor interactions through ligand-induced homo- and hetero-dimer formation. Upon ligand-induced dimerization, these receptors autophosphorylate on specific tyrosine residues in their cytoplasmic domains. The phosphorylated tyrosine residues serve as high affinity docking sites for proteins which possess SH2 or phosphotyrosine binding (PTB) domains. Such proteins include Shc, Grb2 and the p85 subunit of phosphoinositide 3'-kinase (PI 3-kinase). This leads to activation of signalling pathways such as the mitogen-activated protein kinase pathway, resulting in a complex cascade of intracellular signals culminating in specific events for target cells.

One of the most thoroughly investigated subfamilies of RTKs is the ErbB family, which encompasses four known receptors: ErbB-1 (also called epidermal growth factor receptor (EGFR)), ErbB-2 (also called HER2 or Neu), ErbB-3 and ErbB-4 (Alroy and Yarden, 1997, FEBS Letters 410: 83-86). These receptors are widely distributed throughout different tissues, and, depending on cell type and physiological conditions, act to mediate growth inhibition or induction of cellular proliferation and differentiation.

Many peptide growth factors are ligands for the ErbB receptor family. This group of factors shares a high degree of sequence similarity, particularly with respect to a common six-cysteine 36-40 amino acid residue epidermal growth factor (EGF) motif. This motif has a spacing of $CX_7CX_4CX_{10}CX_1CX_8C$, and forms three intramolecular disulfide bonds (C_1-C_3, C_2-C_4, C_5-C_6) and a characteristic three loop

structure, including the C_1-C_3 disulphide loop, C_2-C_4 disulphide loop and C_5-C_6 disulphide loop.

Mammalian ligands for the ErbB family include EGF (Savage *et al.*, 1972, J. Biol. Chem. 247: 7612-7621), transforming growth factor-α (TGF-α) (Marquardt *et al.*, 1984, Science, 223: 1079-1082), heparin-binding EGF-like growth factor (HB-EGF) (Higashiyama *et al.*, 1991, Science, 251: 936-939), epiregulin (Toyoda *et al.*, 1995, J. Biol. Chem., 270: 7495-7500), amphiregulin (Shoyab *et al.*, 1989, Science, 243: 1074-1076), neural- and thymus-derived activator for ErbB kinases (NTAK) (Higashiyama *et al.*, 1997), the neuregulin (NRG) subfamily, which includes the products of four genes (NRG1 (Marchionni *et al.*, 1993, Nature, 362: 312-318), NRG2 (Chang *et al.*, 1997, Nature, 387: 509-512; Carraway *et al.*, 1997, Nature, 387: 512-515), NRG3 (Zhang *et al.*, 1997, Proc. Natl. Acad. Sci. (USA), 94: 9562-9567) and NRG4 (Harari *et al.*, 1999, Oncogene, 18: 2681-2689)), and betacellulin (BTC) (Shing *et al.*, 1993, Science, 259: 1604-1607).

BTC was originally purified from the conditioned medium of a mouse pancreatic β-cell carcinoma (insulinoma) cell line, as a 32 kDa glycoprotein with mitogenic activity for fibroblasts, retinal pigment epithelial cells and smooth muscle cells (Shing *et al.*, 1993, Science, 259: 1604-1607). Human BTC has been cloned from the human breast cancer cell line MCF-7 (Sasada *et al.*, 1993, Biochem. Biophys. Res. Commum. 190: 1604-1607) and bovine BTC has been cloned from a bovine kidney cell line (Dunbar *et al.*, 1999, Biochem. J. 344, 713-721). International Patent Application No. PCT/US 5229493 and European Patent Application 0555785 A1 disclose the amino acid sequence of mammalian BTC and methods for its production by recombinant DNA technology. However, hitherto a naturally occurring splice variant of BTC has not been disclosed.

BTC is synthesised as a membrane-anchored precursor protein which can be proteolytically cleaved to release the soluble mature growth factor. Mature BTC binds

to and activates the erbB1 and erbB-4 homodimers, and in addition, all possible ErbB heterodimers, including the highly oncogenic heterodimeric erbB2-erbB3 receptor complex (Beerli and Hynes, 1996, J. Biol. Chem., 271: 6071-6078; Riese *et al.*, 1996, Oncogene,, 12: 345-353; Alimandi *et al.*, 1997, EMBO J., 16: 5608-5617; Wang *et al.*, 1998, Proc. Natl. Acad. Sci. (USA), 95: 6809-6814; Pinkas-Kramarski *et al.*, 1998, Oncogene, 16: 1259-1258; Jones *et al.*, 1999, FEBS Letters, 447: 227-231).

BTC is expressed in a wide range of normal tissues, and is particularly highly expressed in both fetal and adult pancreas (Watanabe *et al.*, 1994, J. Biol. Chem., 269: 9968-9973). BTC has been shown to be overexpressed in a human pancreatic cancer (Yokoyama et al.,. 1995, Int. J. Oncology, 7: 825-829). Recent studies suggest that BTC may play an important role in the formation of pancreatic β-cells (Mashima *et al.*, 1996, J. Clin. Invest., 97: 1647-1654; Watada *et al.*, 1996, Diabetes, 45: 1828-1831; Huotari *et al.*, 1998, Endocrinology, 139: 1494-1499; Ishiyama *et al.*, 1998, Diabetologia, 41: 623-628; Mashima *et al.*, 1999, Diabetes, 48: 304-309).

In addition to their role in cellular proliferation and differentiation, members of the ErbB receptor family have been implicated in the development of a variety of human carcinomas (Gullick, 1991, Br. Med. Bull. 47: 87-98; Hynes and Stern, 1994, Biochim. Biophys. Acta. 1198: 165-184; Salomon *et al.*, 1995, Crit. Rev. Oncol. Hematol., 19: 183-232). These receptors are thought to contribute to tumorigenesis as a result of their overexpression due to gene amplification or enhanced transcription of the ErbB oncogene. Overexpression or altered expression of ErbB1 has been reported for glioblastomas, epidermoid carcinomas, breast carcinomas and other tumor types. Moreover, overexpression of ErbB2 is associated with aggressive breast cancers, and generally predicts a poor short-term clinical outcome (Slamon *et al.*, 1987, Science, 235: 177-182; Hynes, Sem. Cancer Biol., 4:

19-26 1993). Although ErbB3 overexpression in human tumors has not been as frequently observed, ErbB3 has been found to be overexpressed and/or constitutively activated in a subset of mammary tumor cell lines (Alimandi *et al.*, 1995, Oncogene, 10: 1813-18211995).

ErbB receptors may therefore have a central role in both the development and progression of human tumors. Thus these receptors and their ligands are viewed as potential therapeutic targets for the development of drugs which interfere with receptor activation. In particular it is desirable to develop an ErbB receptor antagonist, such as an ErbB ligand-like protein which has receptor binding affinity, but is unable to activate the receptor. Although much progress has been made in elucidating the requirements for interaction of ErbB ligands with the ErbB receptors, attempts to design such an antagonist have as yet been unsuccessful. It is an object of the invention to overcome, or at least alleviate the shortcomings of the prior art.

SUMMARY OF THE INVENTION

Accordingly, in a first aspect the invention provides an isolated polynucleotide molecule selected from the group consisting of

(a) a polynucleotide molecule which encodes a naturally-occurring splice variant of BTC, in which nucleotide sequences encoding the last C_5-C_6 disulphide loop which are normally present in the gene which encodes authentic BTC are absent;

(b) a polynucleotide molecule which has a sequence substantially homologous to the molecule of (a), which encodes a polypeptide which has the ability to bind to an ErbB receptor; and

(c) an analogue, fragment, mutant, derivative, or allelic variant of the molecule of (a), which encodes a polypeptide which has the ability to bind to an ErbB receptor.

The splice variant of the invention is designated BTC-β, and polynucleotides encoding BTC-β are referred to

as BTC-β polynucleotides. The term "authentic" when used with reference to BTC means the normal soluble mature BTC protein.

The term "substantially homologous" sequence refers to a polynucleotide which has a sequence which encodes a polypeptide which is functionally equivalent to the specific BTC-β sequences disclosed herein, and encompasses substitutions, deletions and insertions in the specifically-disclosed polynucleotide sequences.

The variant of the polynucleotide may be a naturally occurring allelic variant of the polynucleotide or a non-naturally occurring variant of the polynucleotide. As is known in the art, an allelic variant is an alternative form of a polynucleotide sequence which may have a substitution, deletion or addition of one or more nucleotides, which does not substantially alter the function of the encoded polypeptide.

Preferably the polynucleotide molecule has a sequence of human origin.

The molecule may be DNA or RNA. The DNA may be genomic DNA or cDNA. The molecule may be naturally occurring or recombinant, and may be isolated from a cellular source, or may be chemically synthesized. Suitable methods of molecular cloning or of chemical synthesis are well known in the art.

The DNA may be double stranded or single stranded, and if single stranded may be the coding (sense) or non-coding (anti-sense) strand. The coding sequence which encodes the polypeptide may be identical to the coding sequence shown in Figure 2, or may be a different coding sequence which, as a result of the redundancy of the genetic code, encodes the same polypeptide.

It will be clearly understood that the invention also encompasses a nucleic acid molecule capable of hybridizing to the coding sequence shown in Figure 2, under at least low stringency hybridization conditions, or a nucleic acid molecule with at least 70% sequence identity

to SEQ ID NO.6. Methods for assessing ability to hybridize and percentage sequence identity are well known in the art. For example, a suitable program for determining percentage sequence identity is *BLAST 2.0 Sequence Comparison (NIH)* (http://www.ncbi.nlm.nih.gov/blast/b12seq/b12.html). Preferably, the limiting parameters imposed for such a task are the default setting for the program as displayed on this web site. Even more preferably the nucleic acid molecule is capable of hybridizing to the coding sequence shown in Figure 2 under high stringency conditions, or has at least 80%, most preferably at least 90% sequence identity thereto. A nucleic acid molecule having at least 70%, preferably at least 90%, more preferably at least 95% sequence identity to one or more of these sequences is also within the scope of the invention.

The invention also includes polynucleotides in which the coding sequence for the polypeptide is fused in frame to a polynucleotide sequence which aids in expression of a polypeptide from a host cell, for example a polynucleotide leader sequence encoding the first 11 or 46 amino acids of pig growth hormone or the first 105 amino acids of thioredoxin. Other suitable leader sequences are known in the art.

The polynucleotides of the invention may also have the coding sequence fused in frame to a marker sequence which aids in purification of the polypeptides of the invention. For example, the marker sequence may be a hexa-histidine tag to provide for purification of the polypeptide fused to the marker in the case of a bacterial host or may be a Flag™ (Kodak) tag when a mammalian host, e.g. human embryonal kidney 293-T cells, is used.

One particularly preferred embodiment of this aspect of the invention provides a polynucleotide sequence, which may be DNA or RNA, which encodes

(a) a polypeptide having a deduced amino acid sequence as set out in any one of SEQ ID NO 1, NO 2, NO 3, NO 4, NO 5 or NO 6, or

(b) a fragment, analogue, derivative or allelic variant thereof which has the ability to bind to an ErbB receptor,

and which optionally also comprises one or more additional coding or non-coding sequences.

The polynucleotide sequence may comprise the complete coding sequence (SEQ ID No. 1), or may optionally comprise

(a) only the coding sequence for the putative mature polypeptide (amino acids 32-94, SEQ ID No. 2);

(b) the coding sequence for the mature polypeptide, and additional coding sequences, such as a leader or secretory signal sequence (amino acids 1-94, SEQ ID No. 3); the coding sequence for the mature polypeptide and other additional coding sequences (for example amino acids 32-129, SEQ ID No. 4, or amino acids 32-111, SEQ ID No. 5); or

(c) the coding sequence for the mature polypeptide (and optionally additional coding sequence as described above) and non-coding sequence, such as introns or non-coding sequence situated 5' and /or 3' of the coding sequence.

For example, the first 93 nucleotides of the BTC-β polynucleotide encode amino acids which represent the signal peptide which is cleaved to form the mature polypeptide, for example amino acids 32-129 (SEQ ID No.4).

In addition to therapeutic applications, the BTC-β polynucleotides of the invention have wide utility, including but not limited to their use in the preparation of BTC-β expression vectors, primers and probes to detect and clone BTC-β, and diagnostic reagents. Diagnostic methods utilizing BTC-β polynucleotides include hybridisation and PCR assays utilising BTC-β polynucleotides as primers or probes, as appropriate.

In a second aspect, the invention provides an isolated polypeptide molecule which is a naturally occurring splice variant of human BTC, or an analogue,

fragment or derivative thereof which has the ability to bind to an ErbB receptor.

Preferably the polypeptide has the ability to act as an agonist or an antagonist to one or more activities mediated by ErbB receptors. A person skilled in the art will readily be able to determine the biological activity of a given splice variant of the invention, using methods known in the art.

The terms "fragment", "analogue", "mutant" and "derivative" when referring to the polypeptide of the invention mean a molecule which retains essentially the same biological function or activity as this polypeptide. Thus an analogue includes a proprotein which can be activated by cleavage of the proprotein portion to produce an active mature polypeptide.

Thus a variant BTC-β amino acid sequence is included within the scope of the invention, provided that it is functionally active. As used herein, the terms "functionally active" and "functional activity" in reference to BTC-β means that the BTC-β is able to bind to an ErbB receptor, and/or that the BTC-β is immunologically cross-reactive with an antibody directed against an epitope of naturally-occurring BTC-β. Therefore, BTC-β amino acid sequence variants generally will share at least about 75%, preferably greater than 80%, and more preferably greater than 90% sequence identity with the translated amino acid sequence set out in SEQ ID NO: 2, after aligning the sequences to provide for maximum homology, as determined, for example, by the Fitch, et al., *Proc. Nat. Acad. Sci. USA* 80:1382-1386 (1983), version of the algorithm described by Needleman, et al., *J. Mol. Biol.* 48:443-453 (1970). Amino acid sequence variants of BTC-β are prepared by introducing appropriate nucleotide changes into BTC-β DNA, and subsequently expressing the resulting modified DNA in a host cell, or alternatively may be prepared by *in vitro* synthesis. Such variants include deletions, insert BTC-β or substitutions of amino acid residues within the BTC-β

amino acid sequence set out in SEQ ID NO: 2. Any combination of deletion, insertion, and substitution may be made to arrive at an amino acid sequence variant of BTC-β, provided that the variant possesses the desired functional characteristics described herein. Changes made in the amino acid sequence set out in SEQ ID NO: 2 to arrive at an amino acid sequence variant of BTC-β also may result in further modifications of BTC-β when it is expressed in host cells, for example, by virtue of such changes introducing or moving sites of glycosylation.

In a preferred embodiment, this aspect of the invention relates to BTC-β polypeptides which have the deduced amino acid sequences set out in Figure 4, as well as fragments, analogues, and derivatives of such polypeptides.

The polypeptide of the invention may be a recombinant polypeptide, a natural polypeptide or a synthetic polypeptide, and is preferably a recombinant polypeptide.

The fragments, analogues, and derivatives of the polypeptides in Figure 4 include those in which:

(i) one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue, preferably a conserved amino acid residue; such a substituted amino acid residue may or may not be one encoded by the genetic code,

(ii) one or more of the amino acid residues includes a substituent group,

(iii) the mature polypeptide is fused with another compound to increase the half-life of the polypeptide, or

(iv) additional amino acids, such as a sequence which is employed for purification of the mature polypeptide, are fused to the mature polypeptide.

Such fragments, analogues and derivatives are deemed to be within the scope of the invention.

The polypeptides and polynucleotides of the

invention are preferably provided in an isolated form, and are preferably purified to homogeneity.

In a third aspect, the invention provides a vector comprising a polynucleotide molecule according to the invention, and host cells transformed by such vectors. The host cell may be prokaryotic or eukaryotic. Preferably the vector is an expression vector. Suitable host cells include bacterial, yeast, insect, and mammalian cells.

In a fourth aspect, the invention provides a method of producing BTC-β, comprising the steps of transforming an expression vector according to the invention into a suitable host cell, cultivating the host cell under conditions suitable for expression of BTC-β, and isolating BTC-β.

It will be appreciated that host cells comprising selected constructs so formed may express the BTC-β as a fusion protein within inclusion bodies (IB). By the term "BTC-β fusion protein" we mean a polypeptide consisting of two linked protein components, one of which is selected so as to be expressed in the host cell under the control of a suitable promoter, and the other of which comprises the polypeptide bioactive factor incorporating the motif that confers BTC-β activity. The fusion protein is produced in order to facilitate the expression and/or processing of the amino acid sequence of the BTC-β activity. Preferably the BTC-β fusion protein is produced by an appropriate host cell in a fermenter by conventional means understood by those skilled in the art.

Preferably the BTC-β is isolated from the host cell following disruption of the host cell by homogenisation, and processed to its biologically pure form using conventional methods of protein purification well recognised by those skilled in the art. These include oxidative refolding to achieve correct disulphide bonding, chemical cleavage of the fusion partner (if used) from the BTC-β, and various chromatographic steps. The BTC-β may be isolated as a biologically pure form of the fusion protein,

and may then be cleaved from its fusion partner, yielding a peptide that is not extended.

In a fifth aspect, the invention provides an antibody directed against a polypeptide according to the invention, or a fragment or derivative of such an antibody which retains its ability to bind to BTC-β. It will be clearly understood that the antibody of the invention may be polyclonal or monoclonal, or may be a $(Fab)_2$ fragment, a Fab fragment, a Fv fragment, or a ScFV fragment. Methods for preparation of polyclonal and monoclonal antibodies and preparation of the aforesaid fragments are very well known in the art.

In a sixth aspect, the invention provides a composition comprising a polynucleotide molecule, polypeptide molecule or antibody according to the invention, together with a pharmaceutically-acceptable carrier. The composition may optionally further comprise one or more additional therapeutic agents, such as cytotoxic agents or inhibitors of ErbB receptor activation.

While it is particularly contemplated that the compounds of the invention are suitable for use in medical treatment of humans, they are also applicable to veterinary treatment, including treatment of companion animals such as dogs and cats, and domestic animals such as horses, cattle and sheep, or zoo animals such as felids, canids, bovids, and ungulates.

Methods and pharmaceutical carriers for preparation of pharmaceutical compositions are well known in the art, as set out in textbooks such as Remington's Pharmaceutical Sciences, 19th Edition, Mack Publishing Company, Easton, Pennsylvania, USA.

The compounds and compositions of the invention may be administered by any suitable route, and the person skilled in the art will readily be able to determine the most suitable route and dose for the condition to be treated. Dosage will be at the discretion of the attendant physician or veterinarian, and will depend on the nature

and state of the condition to be treated, the age and general state of health of the subject to be treated, the route of administration, and any previous treatment which may have been administered.

The carrier or diluent, and other excipients, will depend on the route of administration, and again the person skilled in the art will readily be able to determine the most suitable formulation for each particular case. The carrier to be used and the dose and route of administration will depend on the nature of the condition to be treated and the age and general health of the subject, and will be at the discretion of the attending physician or veterinarian.

According to a seventh aspect, the invention provides a method of treatment of a condition mediated or modulated by ErbB receptors, comprising the step of administering an effective amount of a composition according to the invention to a subject in need of such treatment. Preferably the condition is associated with overexpression of an ErbB oncogene.

In one preferred embodiment the condition relates to cellular proliferation and/or differentiation. More preferably the condition is a hyperproliferative disease such as a cancer. Most preferably the cancer is selected from the group consisting of breast cancer, epidermoid carcinoma, glioblastoma, and pancreatic cancer.

The BTC-β polypeptide of the invention may be employed to stimulate the growth, proliferation and/or differentiation of mammalian cells, such as epithelial cells, and more organised structures, such as skin *in vitro* or *in vivo*, either alone or in combination with other factors.

In another preferred embodiment, this aspect of the invention provides a method of promoting mitogenesis in a subject in need of such treatment, comprising the step of administering an effective amount of the composition according to the invention to the subject. Preferably this

embodiment provides a method of promoting cell replication, for example in stimulating wound repair.

Thus the BTC-β polypeptide of the invention may be employed as an agent for treatment of wound healing and/or to stimulate tissue repair. A wide range of wounds may be treated by the polypeptide, including cutaneous wounds, burns, corneal wounds, and injuries to epithelial cell-lined hollow organs of the body. Other conditions suitable for treatment with the polypeptide of the invention include chronic conditions, such as chronic ulcers, diabetic ulcers and other non-healing (trophic) conditions.

In a third embodiment, it is contemplated that, because it appears that BTC may play an important role in the formation of pancreatic β-cells, the polypeptide of the invention may also be used for the treatment of diabetes.

It will be appreciated that the composition of the invention may be administered in conjunction with one or more additional therapeutic agents.

It is contemplated that because of the activity of BTC, BTC-β will find utility as a replacement for serum or for serum components in media for mammalian cell culture.

It will be clearly understood that the invention includes within its scope variant forms of other members of the EGF family of peptide growth factors which are capable of acting as ligands for the ErbB family of receptors, herein defined as "analogous growth factor variants", in which amino acid residues normally present in the authentic polypeptide are absent, in a manner analogous to their absence from BTC-β. Preferably the C_5-C_6 disulphide loop normally present in the authentic molecule is absent, in a manner analogous to BTC-β. Suitable authentic polypeptides selected from the EGF family include epidermal growth factor, transforming growth factor-α, heparin-binding EGF-like growth factor, epiregulin, amphiregulin, neural and thymus-derived activator for ErbB kinases (NTAK), and

members of the neuregulin subfamily (NRG1, NRG2, NRG3 and NRG4). It is expected that such variant forms will function in a manner analogous to that found in the BTC-β polypeptides of the invention. It will also be appreciated that, once knowing the sequence variation disclosed herein, both BTC-β and analogous variants of other growth factors may be synthesised by chemical means such as solid phase polypeptide synthesis or by recombinant methods, all of which are well known in the art.

The invention also encompasses BTC-β from non-human mammals; the BTC-β polynucleotides of the invention may readily be used as probes for isolation of corresponding polynucleotides from cells of other mammals using methods which are routine in the art.

For the purposes of this specification it will be clearly understood that the word "comprising" means "including but not limited to", and that the word "comprises" has a corresponding meaning.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A shows the detection of authentic human BTC and splice variant human BTC-β in MCF-7 cells (Lane 1) and human breast skin fibroblasts (Lane 2) by RT-PCR. Lane 3 is a control without cDNA template. Figure 1B shows the corresponding Southern blot using a cDNA probe encompassing polynucleotides encoding amino acids D^{32}-Y^{111} of human BTC.

Figure 2A compares a partial nucleotide and deduced amino acid sequence (in one letter amino acid code) of authentic human BTC and splice variant human BTC-β. The RT-PCR products obtained from MCF-7 cDNA in Figure 1A were cloned into pBluescript II SK and the inserts sequenced. Sequences surrounding the point of the 147 bp deletion (indicated by a downward arrow) are shown. Figure 2B shows the complete nucleotide sequence and deduced amino acid sequence of BTC-β cDNA.

Figure 3 compares the complete amino acid sequences of authentic human BTC and splice variant human

BTC-β in a hypothetical alignment. The horizontal dashes within the BTC-β sequence indicate the sites of the missing amino acids when compared to the authentic hBTC sequence. The schematic below shows the overall structure of authentic human BTC compared to BTC-β.

Figure 4 shows the amino acid sequences of the BTC-β polypeptides ($BTC\beta_{1-129}$, $BTC\beta_{1-94}$, $BTC\beta_{32-94}$, $BTC\beta_{32-129}$, $BTC\beta_{32-111}$ and $BTC\beta_{95-129}$).

Figure 5 shows the expression of authentic human BTC (563 bp) and BTC-β (416 bp) in a variety of normal human tissues by RT-PCR and Southern blot analysis.

Figure 6 shows an SDS-PAGE gel showing the bacterial expression pET3.2a BTC-β constructs. Lane 1, molecular weight markers; Lane 2, BTC^{32-111} thioredoxin fusion protein, Lane 3, BTC^{32-94} thioredoxin fusion protein; Lane 4, BTC^{32-129} thioredoxin fusion protein; Lane 5, molecular weight markers.

DETAILED DESCRIPTION OF THE INVENTION

The invention will now be described in detail by way of reference only to the following non-limiting examples, and to the figures.

The polynucleotide sequence of the invention was isolated from MCF-7 cells. It contains an open reading frame encoding a polypeptide of 129 amino acids. The polynucleotide sequence is identical to that of hBTC, except for a 147 bp deletion within the open reading frame (encoding 49 amino acids)resulting in the absence of the C_5-C_6 disulphide loop normally present in the EGF domain (See Figure 2B and 3): This was generated as a result of alternative mRNA splicing (exon skipping) of one of the exons of the human BTC gene.

BTC-β polynucleotides may be obtained from a variety of cell sources which express BTC-β encoding mRNA. The inventors have identified a number of suitable human cell sources for BTC-β polynucleotides, including but not limited to kidney, liver, pancreas, and a variety of breast

carcinoma cell lines (e.g., MCF-7).

For example, polynucleotides encoding BTC-β polypeptides may be obtained by cDNA cloning from RNA isolated and purified from cell sources. cDNA libraries of clones may be prepared using techniques well known to those in the art, and may be screened for BTC-β encoding DNA with nucleotide probes which are substantially complementary to any portion of the BTC gene. Various PCR cloning techniques may also be used to obtain the BTC-β polynucleotides of the invention.

Thus polynucleotides encoding BTC-β polypeptides of the invention may be obtained by PCR, using oligonucleotide primers comprising polynucleotide sequences encoding portions of the BTC gene. Preferably the primer comprises the extreme 5' and 3' coding regions. More preferably the oligonucleotide primers have the following sequences, or sequences substantially homologous thereto (SEQ ID No. 8):

5' GAGCGGGGTTGATGGACCGG 3'

5' TTAAGCAATATTTGTCTCTTC 3'

Representative low and high stringency conditions of hybridisation as referred to herein are as follows: High stringency: hybridization at 42°C in 50% formamide, 3 x SSC, 0.1% SDS, 20 x Denhardt's, 50 μg/ml salmon sperm DNA overnight and washed with a final wash of 0.1 x SSC, 0.1% SDS at 42°C.

Low stringency: hybridization at 28°C in 50% formamide, 3 x SSC, 0.1% SDS, 20 x Denhardt's, 50 μg/ml salmon sperm DNA overnight and washed with a final wash of 0.1 x SSC, 0.1% SDS at room temperature.

Host cells are transformed or transfected with the vectors of this invention, for example a cloning vector or an expression vector. Various expression vector/host systems may be utilised equally well by those skilled in the art for the recombinant expression of BTC-β polypeptides. Such systems include, but are not limited to micro-organisms such as bacteria transformed with

recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors comprising the desired BTC-β polynucleotide coding sequence; yeast transformed with recombinant yeast expression vectors comprising the desired BTC-β polynucleotide coding sequence; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) comprising the desired BTC-β polynucleotide coding sequence; or animal cell systems transfected with appropriate mammalian expression vectors comprising the desired BTC-β polynucleotide coding sequence.

The appropriate DNA sequence may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) by procedures well known to those skilled in the art.

The DNA sequence inserted in the expression vector is operatively linked to an expression control sequence(s) (promoter) to direct mRNA synthesis. Depending on the host/vector system utilised, any one of suitable transcription/translation elements may be used. For instance, when cloning in prokaryotic cells (*E. coli*) the trc or T7 promoter may be used; when cloning in mammalian expression systems, promoters isolated from the genome of mammalian cells (e.g., mouse metallothionein promoter) or from viruses that grow in these cells (e.g., human cytomegalovirus immediate-early (CMV) promoter) may be used. Promoters produced by recombinant DNA or synthetic techniques may also be used to provide transcription of the inserted sequences. The expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. Specific initiation signals are also required for efficient translation of inserted coding sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where the entire BTC-β coding sequence, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vectors, no additional translational control

signals may be needed. However, in cases where only a portion of the BTC-β coding sequence is inserted, exogenous translational control signals, including the ATG initiation codon, must be provided.

Furthermore, the initiation codon must be in phase with the reading frame of the BTC-β coding sequences to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of transcription attenuation sequences, enhancer elements etc.

In addition, it is preferable that the expression vectors comprise one or more selectable marker genes to provide a phenotypic trait for selection of transformed or transfected host cells, such as neomycin (G418) resistance for eukaryotic cells, or ampicillin resistance for prokaryotic cells such as *E. coli*.

The vector containing a DNA molecule of the invention, as well as an appropriate promoter or control sequence, may be employed to transform or transfect an appropriate host to permit the host to express the protein.

Representative examples of appropriate hosts include, but are not restricted to, bacterial cells, such as *E. coli*; insect cells such as *Drosophila* and Sf9; animal cells such as CHO, COS, or 293 cells.

More particularly, the invention also includes recombinant constructs comprising one or more of the sequences as broadly described above. The constructs comprise a vector, such as a plasmid, into which a sequence of the invention has been inserted, in a forward or reverse orientation. Preferably the construct further comprises one or more regulatory sequences, such as a promoter, operably linked to the sequence. Many suitable vectors and promoters are known to those skilled in the art.

In a further embodiment, the invention relates to

host cells containing the above-described constructs. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or a prokaryotic cell, such as a bacterial cell. Introduction of the construct into the host cell can be via a variety of methods which are well known to those skilled in the art.

The constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence. Alternatively, the polypeptide of the invention can be synthetically produced by conventional peptide synthesisers.

The polypeptide of the invention, produced in a variety of different vector/host expression systems as described above, can be recovered and purified from recombinant cell cultures by a wide variety of methods, including ammonium sulphate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxyapatite chromatography, and reverse-phase high performance liquid chromatography (HPLC). Protein refolding steps can be used, as necessary, in completing the configuration of the desired polypeptide.

The polypeptide of the invention may be a naturally purified product, or a product of chemical synthetic procedures, or produced by recombinant techniques using a prokaryotic or eukaryotic host. Depending upon the host employed in a recombinant production procedure, the polypeptides of the invention may be glycosylated or may be non-glycosylated.

The BTC-β polypeptide of the invention may be employed as research agents and materials for discovery of treatments and diagnostics for human diseases. Thus one suitable method of using the BTC-β polypeptide as a diagnostic for human disease comprises the step of quantitatively determining the amount of BTC-β polypeptide present in a sample from a subject, and comparing the

amount thus determined with the amount present in a sample from a normal subject, in which the presence of a significantly different amount in the test subject indicates the presence of a disease.

The BTC-β polypeptide may have the ability to mediate or modulate ErbB receptor activity. The person skilled in the art will readily be able to determine the biological activity of the BTC-β polypeptide mediated by ErbB receptors, using methods known in the art.

For example, the methylene blue cell proliferation assay may be used for the characterisation of the cellular proliferation activity of BTC-β polypeptides. Cell lines suitable for this assay include, but are not limited to, Balb/c 3T3, HaCaT, IEC-6, SF3169, Mv1Lu and CalOst cells. Preferably the cell lines are subcultured at a density of 10-20 x 10^4 cells/ml^{-1} and incubated overnight at 37^0C in the presence of 5% CO_2. The cells are then washed extensively with DMEM to remove any residual medium, after which BTC-β can be added at various concentrations. Authentic BTC can optionally be assayed in parallel for comparison. After incubation for 48 h, the plates are washed twice with 0.15M NaCl, followed by the quantitation of the cell mass by the addition of 1% methylene blue and measurement of absorbance at 600nm.

Furthermore, a radio-receptor assay may be used to characterise the competition of BTC-β for ErbB receptors with other growth factors. For example, BTC-β polypeptides can be analysed by the competitive displacement of ^{125}I-betacellulin from receptors present on various cell lines including, but not limited to, AG2804 fibroblasts and Chinese hamster ovary cells transfected with ErbB-2, ErbB-3 and ErbB-4 receptor homodimers. Preferably cells are grown to 70-80% confluence in DMEM supplemented with 10% (v/v) foetal bovine serum in 24-well plates. The cells are washed twice with binding buffer; [100 mM Hepes (pH 7.6), 120 mM NaCl, 5 mM KCI, 1.2 mM MgSO4.7H_2O, 8 mM glucose, 0.1% bovine serum albumin] and then incubated with appropriate

concentrations of each peptide and ^{125}I-betacellulin in binding buffer for 18 h at 4°C. At the end of this period, cells are washed three times with Hanks' buffered salt solution and lysed with 1 ml of 0.5 M NaOH, 0.1 % (v /v) Triton X-100 for 30 min at room temperature. Incorporation of radioactivity into the cell lysate is determined with a γ-counter; total binding is determined in the absence of competing peptides.

BTC-β polypeptide may be incorporated in physiologically or pharmaceutically-acceptable carriers to provide compositions for application to the affected area. The nature of the carriers may vary widely, and will depend on the condition to be treated and the intended site of application. For application to the skin, a cream or ointment base is preferred. In addition, the BTC-β polypeptides of the invention may be impregnated into bandages and other wound dressings to provide for continuous exposure of the wound to the polypeptide.

The concentration of polypeptide in the treatment composition is not critical but should be an effective amount to induce epithelial proliferation. The term "effective amount" as used herein in all methods of use for BTC-β polypeptides, means an amount sufficient to elicit a statistically significant response at a 95% confidence level ($p<0.05$ that the effect is due to chance alone). The amount of polypeptide employed can be determined empirically, based on the response of cells *in vitro* and response of experimental animals to the subject polypeptide or formulations containing the subject polypeptide.

Additionally, the polypeptide of the invention may be used to prevent or inhibit proliferation of adenocarcinoma cell proliferation. For example, many cancer cells overexpress various members of the ErbB receptor family, and the polypeptides of the invention may bind these receptors in such a way that receptor dimerization and subsequent signalling is inhibited. In this way, blocking the action of the ErbB receptors may provide a

method of treatment of cancer. In one specific embodiment, BTC-β polypeptides may be used for systemic and targeted therapy of certain cancers overexpressing ErbB receptors, such as metastatic breast cancer, with minimal toxicity to normal tissues and organs.

In addition it will also be understood by those skilled in the art that the BTC-β polypeptides of the invention can be conjugated with various toxins that are cytotoxic to cancerous cells. More particularly, ErbB-expressing tumor cells may be specifically targeted and killed by contacting such tumor cells with a fusion protein comprising a cytotoxic polypeptide covalently linked to any one of the BTC-β polypeptides of the invention. Examples include, but are not limited to, ricin, daunorubicin, methotrexate and various bacterial toxins, such as *Pseudomonas* exotoxin. Toxin-conjugated-BTC-β polypeptides for targeted cancer therapy may be administered by any route which will result in polypeptide interaction with the target cancer cells, including systemic administration and injection directly to the site of the tumor. Another therapeutic strategy is administration by sustained-release systems, such as semipermeable matrices of solid hydrophobic polymers containing the therapeutic agent. A further therapeutic strategy is the administration of BTC-β polypeptides with an agent which inhibits ErbB receptor activation. Such agents would include antibodies directed to an epitope of the ErbB receptor.

The invention also encompasses polyclonal and monoclonal antibodies which recognise epitopes which are specific to BTC-β polypeptides. Anti-BTC-β antibodies may be used for the detection and quantification of BTC-β polypeptide expression in cultured cells, tissue samples, and *in vivo*. For example, monoclonal antibodies recognising epitopes from different parts of the BTC-β structure may be used to detect and/or distinguish binding from non-binding regions of the ligand. In a preferred embodiment, the antibody is a polyclonal or monoclonal antibody which

recognises a BTC-β specific epitope which comprises amino acid residues 94 and 95 of the BTC-β polypeptide.

Anti-BTC-β antibodies may also be useful in detecting the presence of a disease. Suitable methods using the anti-BTC-β antibodies as a diagnostic for human disease comprise the step of contacting a sample from a human subject with an anti-BTC-β antibody under suitable conditions so as to form a complex between the antibody and an epitope contained within the protein, and comparing the quantitatively determined amount of complex with the amount present in a sample from a normal subject; the presence of a significantly different amount of complex in the sample from the test subject indicates the presence of a disease. Suitable procedures for quantification of the complex will be known to persons skilled in the art, and include, but not are not limited to, enzyme-linked immunosorbent assay (ELISA) or the use of radioactive isotopes.

Anti-BTC-β antibodies may also be useful in treating particular human cancers. Anti-BTC-β antibodies may be used to block signal transduction mediated through ErbB receptors, thereby inhibiting undesirable biological responses. Anti-BTC-β antibodies may also be useful for the stimulation or induction of cellular proliferation, for example in wound healing and/or to stimulate tissue repair. A wide range of wounds may be treated using the anti-BTC-β antibodies, including cutaneous wounds, corneal wounds, and injuries to the epithelial cell-lined hollow organs of the body. Other conditions suitable for treatment by this method include chronic conditions, such as chronic ulcers, varicose ulcers, diabetic ulcers, and other non-healing (trophic) conditions. Anti-BTC-β antibodies may also be employed for the treatment of conditions associated with pancreatic dysfunction, such as diabetes.

In addition to the various diagnostic and therapeutic utilities of anti-BTC-β antibodies, a number of industrial and research applications will be apparent to those skilled in the art, including, for example, the use

of anti-BTC-β antibodies as affinity reagents for the purification of BTC-β polypeptides, and as immunological probes for elucidating the biosynthesis, metabolism and biological functions of BTC-β polypeptides.

Various procedures known in the art may be used for the production of polyclonal antibodies to epitopes of BTC-β polypeptides. For the production of polyclonal antibodies, a number of host animals are acceptable for the generation of anti-BTC-β antibodies by immunisation with one or more injections of a BTC-β polypeptide preparation, including but not limited to rabbits, mice, rats, goats, etc. Various adjuvants may be used to increase the immunological response in the host animal, depending upon the host species, including but not limited to alum, iscoms, or complete or incomplete Freund's adjuvant.

Monoclonal antibodies to epitopes of BTC-β polypeptides may be prepared by using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not restricted to, the original hybridoma technique (Köhler and Milstein, Nature 1975, 256: 495-497) and the human B-cell hybridoma technique (Kosbor *et al*. 1983, Immunology Today, 4:72). Recombinant human or humanised versions of anti-BTC-β monoclonal antibodies are a preferred embodiment for human therapeutic applications. Humanised antibodies may be prepared according to procedures described in the literature (e.g., Verhoeyen *et al*., 1988, Science 239: 1534-1536).

Example 1: Detection of BTC-β by RT-PCR and cloning into pBluescript II SK

Total RNA was isolated from 70-80% confluent MCF-7 cells and human breast skin fibroblasts using an RNeasy Mini Kit according to the manufacturer's instructions (Qiagen, Clifton Hill, Vic., Australia). Normal human breast skin fibroblasts were prepared from a piece of skin obtained during surgery for breast reduction. The skin was

cultured as an explant for 5 days in DMEM supplemented with 10% fetal calf serum and penicillin-streptomycin sulphate until the fibroblasts had grown into a monolayer. Total RNA (2 μg) was subsequently reverse transcribed to cDNA with Superscript II enzyme according to the manufacturer's instructions using oligo dT primers (Gibco BRL, Gaithersburg, MD). cDNA corresponding to human BTC-β was amplified by PCR with sense primer

(5'-CTCGTCGACGAGCGGGGTTGATGGACCGG-3') and

antisense primer

(5'-CTCCTGCAGTTAAGCAATATTTGTCTCTTC-3').

Underlined nucleotides correspond to *Sal*I (sense primer) and *Pst*I (antisense primer) restriction sites. PCR was carried out in 50μl of 60 mM Tris-SO_4, 18 mM $(NH_4)_2SO_4$, 1.5 mM $MgSO_4$ (pH 9.1), 0.2 mM dNTPs, 200 ng each primer, 1 U eLONGase (Gibco BRL, Gaithersburg, MD) and 1 μl cDNA. Following an initial incubation at 94°C for 3 min, 35 cycles of amplification were carried out as follows: 94°C for 1 min, 50°C for 1 min and 68°C for 1 min followed by a final 4 min extension at 68°C. The 416 bp BTC-β PCR product was separated on a 2% agarose gel and visualized under ultraviolet light following staining with ethidium bromide. PCR Markers (Promega, Madison, WI) were used as the size standard (Figure 1A). Following electrophoresis the gels were Southern transferred to positively-charged nylon membrane (Roche, Castle Hill, NSW, Australia) in 0.4 M NaOH. The blot was incubated in prehybridization buffer (5x SSC, 5x Denhardt's, 0.1% sodium dodecyl sulphate (SDS), and 50 μg/ml salmon sperm DNA) at 55°C for 2 h and hybridized for 16 h in the same buffer containing ^{32}P-dCTP-labelled human BTC cDNA probe (D^{32}-Y^{111}) generated by the random priming method using a Gigaprime kit (Geneworks, Adelaide, Australia). Two washes were performed in 2x SSC, 0.1% SDS for 5 min at room temperature, followed by two more 15 min washes in 0.5x SSC, 0.1% SDS at 55°C. The blot was then exposed to Kodak XAR X-ray film at -80°C (Figure 1B).

BTC and BTC-β PCR products were cloned and

sequenced by separating the products by agarose gel electrophoresis and recovering the fragments following gel extraction with a Concert kit (Gibco BRL, Gaithersburg, MD). Purified PCR products were then digested with *Sal*I and *Pst*I and subcloned into *Sal*I/*Pst*I-digested pBluescript II SK (Strategene, La Jolla, CA) to generate pBlue-BTC and pBlue-BTC-β, and introduced into *E. coli* JM109. The recombinant plasmids were propagated and sequenced by the dideoxynucleotide chain terminator method, using a Thermosequenase cycle sequencing kit (Amersham-Pharmacia Biotech, Sydney, Australia) with M13 T7 and T3 promoter primers. PCR products were sequenced in both directions to confirm the sequence; this was done twice from two independent clones (Figure 2A and B).

Example 2: Tissue specific expression of BTC-β

To examine the tissue distribution of BTC and BTC-β, a panel of eight normalized cDNA preparations from different adult tissues (Human Multiple Tissue cDNA (MTC) Panel 1; Clontech, CA) were used in PCR as described in Example 1 above. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) specific 5' and 3' primers (sense: 5' were used as a control to verify the presence of cDNA in each reaction. The results are illustrated in Figure 5. Particularly strong expression was observed in kidney, liver and pancreas, and heart, skeletal muscle and lung were also positive.

Example 3: Bacterial Expression of BTC-β polypeptides

BTC-β polypeptides constituting amino acids 32-94 (BTC-β^{32-94}) (see Figure 3 and Figure 4, polypeptide 3) or 32-129 (BTC-β^{32-129}) (see Figure 3 and Figure 4, polypeptide 4) or BTC constituting amino acids 32-111 (BTC$^{32-111}$)(see Figure 3) were expressed as thiororedoxin fusion proteins in the expression vector pET3.2a. The open reading frame sequence of BTC-β^{32-94} was amplified by PCR using the primer set

5'-CGTCCATGGCTGATGGGAATTCCACCAGAAGT-3' (sense)

and

5'-CGTCTCGAGTCAGACACAGGAGGGCGTCTG-3')

and pBlue-BTC-β (see Example 1) as template. *Nco*I and *Xho*I recognition sites were attached to the sense and antisense primers, respectively. For BTC-β^{32-129} the above sense primer was used in conjunction with the antisense primer

5'-CGTCTCGAGTCATTAAGCAATATTTGTCTCTTC-3'

(*Xho*I site is underlined) and pBlue-BTC-β as template. BTC^{32-111} was amplified with sense primer

5'-CGTCCATGGCTGATGGGAATTCCACCAGAAGT-3'

(see above) and antisense primer

5'-CGTCTCGAGTCAGTAAAACAAGTCAACTGT-3'

(*Xho*I site is underlined), using pBlue-BTC as template (see Example 1). After amplification the PCR products were digested with *NcoI/XhoI* and cloned into *Nco*I/*Xho*I-digested pET3.2a vector. The recombinant plasmids, pET-BTC-β^{32-94}, pET- BTC-β^{32-129} and pET- BTC^{32-111} were maintained in *E. coli* JM109. To express the recombinant polypeptides, purified plasmids were transformed into *E. coli* BL21(DE3) cells, and cells containing the appropriate plasmid were grown in LB medium at 37°C until the OD_{600nm} reached 0.3-0.7. Protein expression was then induced by 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG), and growth was allowed to continue for a further 3 h at 37°C. Expression of recombinant proteins was assessed by SDS-PAGE analysis. Briefly, cell pellets (from 3 ml of culture) were resuspended in 300 µl BugBuster Protein Extraction Reagent (Novagen) and incubated for 5 min. The cell lysates were centrifuged at 14 000 rpm for 5 min and aliquots of the supernatants analysed by SDS-PAGE (See Figure 6).

Example 4: Construction of BTC-β expression plasmid for mammalian expression

Full length BTC-β was cloned into the vector pcDNA3.1 (Invitrogen) to generate expression vectors for the mammalian production of BTC-β as follows. Briefly, pBlue-BTC-β (see Example 1) was digested with *Apa*I and *Bam*HI and the released insert purified following agarose

gel electrophoresis. The digested and purified insert was then cloned into *Apa*I/*Bam*HI-digested pcDNA3.1, and recombinant clones identified following transformation in *E. coli* TOP10 cells (Invitrogen). To generate a "Flag-tagged" pcDNA3.1-BTC-β construct in which the Flag epitope DYKDDDDK is inserted between amino acids S^{35}-T^{36} (See figure 3), the pBlue-BTC-β was used as a template in PCR using the primers

5'-CTCGGGAATTCCGACTACAAGGACGACGATGACAAGACCAGAAGTCCTGAA-3' (sense; underlined nucleotides correspond to an *Eco*RI restriction site; double underlined nucleotides encode the FLAG epitope tag) and

5'-CTCCTGCAGTTAAGCAATATTTGTCTCTTC-3' (antisense; underlined nucleotides correspond to a *Pst*I restriction site). The resulting PCR product was purified and digested with *Eco*RI and *Pst*I and cloned into *Eco*RI/*Pst*1-digested pBlue-BTC-β. Subsequently, the resultant Flag-tagged pBlue-BTC-β construct was digested with *Apa*I/*Bam*H1 and cloned into *Apa*I/*Bam*H1-digested pcDNA3.1 to generate pcDNA3.1-FLAG-BTC-β.

Example 5: Large scale bacterial expression and purification of BTC-β polypeptides

For large-scale bacterial expression, the recombinant plasmids pET-BTC-β^{32-94}, pET-BTC-β^{32-129} and pET-BTC^{32-111} (see example 3) were transformed into the *E. coli* strain BL21*trx*B(DE3)(Novagen). This strain is deficient in the protein thioredoxin reductase, thus favouring disulphide bond formation in the cytoplasm of *E. coli*.

Single colonies containing the appropriate plasmid were inoculated into 50 ml of LB medium containing 100 μg/ml ampicillin, and grown with vigorous shaking overnight at 37°C. The next morning 40 ml of this culture was seeded into 800 ml of LB/amp medium and grown for a further 3h. At this point IPTG was added to a final concentration of 1 mM, and growth was allowed to proceed for a further 3h. Cells were then harvested by centrifugation (4000 rpm, 10min at 4°C) and stored at -80°C

until ready for use.

Cell pellets were lysed by addition of 20 ml BugBuster (Novagen) and 4 mg lysozyme (Boehringer-Mannheim) and incubation at room temperature for 10 min with shaking. The viscosity of the homogenate was reduced by sonication (3 x 10 sec bursts on ice) and then centrifuged (16 000 x g, 20 min, 4°C) to remove insoluble debris. The pH of the supernatant was then adjusted to pH 8.0 with concentrated HCl, and imidazole was added to a final concentration of 10 mM. To isolate BTC fusion proteins, 6 ml of pre-equilibrated Ni-NTA agarose resin (Qiagen) (Equilibration buffer: 50 mM NaH_2PO_4, 0.3 M NaCl, 10 mM imidazole, pH 8.0) was added and incubated at 4°C for 1 h on an orbital shaker. The resin was then pelleted by centrifugation (1500 rpm, 5 min) and washed 4 times in 50 mM NaH_2PO_4, 0.3 M NaCl, 40 mM imidazole, pH 8.0). BTC fusion proteins were then eluted from the resin by four successive washes in 8 ml 50 mM NaH_2PO_4, 0.3 M NaCl, 250 mM imidazole, pH 8.0).

BTC^{32-111} or BTC-$\beta^{32-94,32-129}$ peptides were released from the thioredoxin fusion partner by proteolytic cleavage with recombinant enterokinase. Briefly, the eluted fractions containing BTC^{32-111} or BTC-$\beta^{32-94,32-129}$ fusion proteins (approximately 32 ml) were dialysed against 2 x 8L of 50 mM Tris-Cl, 1 mM CaCl2, 0.1% Tween-20 (pH 7.4) (Spectrapore membrane, 3500 MW cut-off) at 4°C. The dialysed sample was then centrifuged to remove any precipitated material and enterokinase (Invitrogen) added to a final concentration of 0.1 units/20 µg protein. The cleavage reaction was then allowed to proceed for 16 hours at 37°C before termination by adjustment of the pH to 3.0 with conc. trifluoroacetic acid (TFA).

In order to separate and purify the released BTC peptides from the fusion partner, the cleavage reaction mixture was applied to a C4 reverse-phase HPLC column (Aquapore BU-300, 2.1mm x 100 mm,Brownlee Lab) at a flow rate of 0.5 ml/min. The column was washed with 0.1% TFA until OD_{214nm} returned to baseline, and protein was eluted

with a gradient of 0.1% TFA-0.08% TFA/80% CH_3CN over 40 min at 0.5 ml/min. Fractions containing authentic BTC^{32-111} or BTC-$\beta^{32-94,32-129}$ peptides were pooled and lyophilised. The purity of the final preparation(s) was confirmed by SDS-PAGE and electrospray ionisation mass spectroscopy.

It will be apparent to the person skilled in the art that while the invention has been described in some detail for the purposes of clarity and understanding, various modifications and alterations to the embodiments and methods described herein may be made without departing from the scope of the inventive concept disclosed in this specification.

CLAIMS

1. An isolated polynucleotide molecule selected from the group consisting of

(a) a polynucleotide molecule which encodes a naturally-occurring splice variant of BTC, in which nucleotide sequences encoding the last C_5-C_6 disulphide loop which are normally present in the gene which encodes authentic BTC are absent;

(b) a polynucleotide molecule which has a sequence substantially homologous to the molecule of (a), which encodes a polypeptide which has the ability to bind to an ErbB receptor; and

(c) an analogue, fragment, mutant, derivative, or allelic variant of the molecule of (a), which encodes a polypeptide which has the ability to bind to an ErbB receptor.

2. An isolated polynucleotide molecule according to claim 1, which is a naturally occurring or a non-naturally occurring variant of the polynucleotide.

3. An isolated polynucleotide molecule according to claim 1 or claim 2, which has a sequence of human origin.

4. An isolated polynucleotide molecule according to any one of claims 1 to 3, which is a double-stranded or single-stranded DNA.

5. An isolated polynucleotide molecule according to any one of claims 1 to 4, which is a single-stranded sense or anti-sense DNA.

6. An isolated polynucleotide molecule according to any one of claims 1 to 5, which is a cDNA.

7. An isolated polynucleotide molecule according to any one of claims 1 to 4, which is an RNA.

8. An isolated polynucleotide molecule according to any one of claims 1 to 7, having a coding sequence

(a) as set out in SEQ ID NO.2,

(b) which is capable of hybridizing under stringent conditions to a molecule of SEQ ID NO.2, or

(c) having at least 70% sequence identity to SEQ ID NO.2.

9. An isolated polynucleotide molecule according to any one of claims 1 to 8, in which the coding sequence for the polypeptide is fused in frame to a polynucleotide sequence which aids in expression of a polypeptide from a host cell.

10. An isolated polynucleotide molecule according to any one of claims 1 to 8, in which the coding sequence is fused in frame to a marker sequence which aids in purification of the BTC splice variant polypeptide.

11. An isolated polynucleotide molecule according to any one of claims 1 to 8, comprising a sequence encoding a leader or secretory signal sequence, optionally linked to the coding sequence via a nucleotide sequence encoding a cleavable amino acid sequence.

12. An isolated polynucleotide molecule according to any one of claims 1 to 8, comprising a non-coding sequence which is an intron or a non-coding sequence situated 5' and/or 3' of the coding sequence.

13. An isolated polynucleotide molecule according to any one of claims 1 to 12, which encodes

(c) a polypeptide having a deduced amino acid sequence as set out in any one of SEQ ID NO 1, NO 2, NO 3, NO 4, NO 5 or NO 6, or

(d) a fragment, analogue, derivative or allelic variant thereof which has the ability to bind to an ErbB receptor,

and which optionally also comprises one or more additional coding or non-coding sequences.

14. A vector comprising a polynucleotide molecule according to any one of claims 1 to 13.

15. A host cell transformed by a vector according to claim 14.

16. A BTC-β splice variant polypeptide encoded by a polynucleotide molecule according to any one of claims 1 to 13.

17. An isolated polypeptide molecule which is a naturally occurring splice variant of human BTC, or an

analogue, fragment or derivative thereof which has the ability to bind to an ErbB receptor.

18. A BTC-β splice variant polypeptide according to claim 16 or claim 17 which has the ability to act as an agonist or an antagonist of one or more activities mediated by ErbB receptors.

19. A BTC-β splice variant polypeptide according to any one of claims 16 to 18, which has an amino acid sequence as set out in SEQ ID NO 1, NO 2, NO 3, NO 4, NO 5 or NO 6, or a fragment, analogue, derivative or allelic variant thereof which has the ability to bind to an ErbB receptor, and which optionally also comprises one or more additional amino acid sequences.

20. A BTC-β splice variant polypeptide according to claim 19 which is operatively linked to a sequence which assists in purification of the mature polypeptide.

21. A BTC-β splice variant polypeptide according to claim 19 which is operatively linked to another compound to increase the half-life of the polypeptide

22. A BTC-β splice variant polypeptide according to any one of claims 16 to 21, in which the BTC-β splice variant is glycosylated.

23. A BTC-β splice variant polypeptide according to any one of claims 16 to 21, in which the BTC-β splice variant is non-glycosylated.

24. An analogous growth factor variant of a member of the EGF family of polypeptide growth factors which is capable of acting as a ligand for the ErbB family of receptors, in which one or more amino acid residues normally present in the authentic polypeptide are absent.

25. An analogous growth factor variant according to claim 24, in which the C_5-C_6 disulphide loop normally present in the authentic molecule is absent.

26. An analogous growth factor variant according to claim 24 or claim 25, in which the member of the EGF family is selected from the group consisting of epidermal growth factor, transforming growth factor-α, heparin-binding EGF-

like growth factor, epiregulin, amphiregulin, neural and thymus-derived activator for ErbB kinases (NTAK), and members of the neuregulin subfamily (NRG1, NRG2, NRG3 and NRG4.

27. An analogous growth factor variant according to any one of claims 24 to 26, which is glycosylated.

28. An analogous growth factor variant according to any one of claims 24 to 26, which is non-glycosylated.

29. A polynucleotide molecule encoding an analogous growth factor variant according to any one of claims 24 to 28.

30. An antibody directed against a polypeptide according to any one of claims 16 to 23 or an analogous growth factor variant according to any one of claims 24 to 27, or a fragment or derivative of such an antibody which retains its ability to bind to BTC-β.

31. An antibody according to claim 30, which is a monoclonal antibody.

32. An antibody according to claim 29 or claim 30, which recognises a epitope comprising amino acid residues 94 and 95 of BTC-β.

33. A composition comprising a polynucleotide molecule according to any one of claims 1 to 13 or claim 28, a polypeptide molecule according to any one of claims 16 to 23, an analogous growth factor variant according to any one of claims 24 to 27, or an antibody according to any one of claims 30 to 32, together with a pharmaceutically-acceptable carrier.

34. A composition comprising a BTC-β splice variant polypeptide according to any one of claims 16 to 23, together with a pharmaceutically-acceptable carrier.

35. A composition comprising an analogous growth factor variant according to any one of claims 24 to 28, together with a pharmaceutically-acceptable carrier.

36. A composition comprising an antibody according to any one of claims 30 to 32, together with a pharmaceutically-acceptable carrier.

37. A composition for culture of mammalian cells, comprising a BTC-β splice variant polypeptide according to any one of claims 16 to 23, or an analogous growth factor variant according to any one of claims 24 to 28, together with a tissue culture-acceptable carrier.

38. A method of producing BTC-β, comprising the steps of transforming an expression vector according to claim 14 into a suitable host cell, cultivating the host cell under conditions suitable for expression of BTC-β, and isolating BTC-β.

39. A method of isolating a polynucleotide according to any one of claims 1 to 13, comprising the steps of
(a) providing a source of cells having BTC-β polypeptide activity;
(b) treating the cells to obtain mRNA therefrom;
(c) treating the mRNA thus obtained to produce cDNA therefrom; and
(d) amplifying the cDNA thus obtained using oligonucleotide primers.

40. A method according to claim 39, in which the oligonucleotide primers has the sequence shown in SEQ ID No 8 or SEQ ID No 9.

41. A method of treatment of a condition mediated or modulated by ErbB receptors, comprising the step of administering an effective amount of a composition according to any one of claims 33 to 36 to a subject in need of such treatment.

42. A method according to claim 41, in which the condition is associated with cellular proliferation and/or differentiation.

43. A method according to claim 41 or claim 42, in which the condition is a hyperproliferative disease.

44. A method according to claim 43, in which the condition is associated with overexpression of an ErbB oncogene.

45. A method according to any one of claims 41 to 44, in which the composition further comprises

(a) an agent which is cytotoxic to cancerous cells, or

(b) an agent which is an inhibitor of ErbB receptor activation.

46. A method according to any one of claims 41 to 45, in which the condition is a cancer selected from the group consisting of breast cancer, epidermoid carcinoma, glioblastoma, and pancreatic cancer.

47. A method of stimulating the growth, proliferation and/or differentiation of mammalian cells or tissues, comprising the step of using a BTC-β splice variant polypeptide according to any one of claims 14 to 21, either alone or in combination with one or more other growth factors.

48. A method according to claim 47, in which the cells are epithelial cells.

49. A method according to claim 48, in which the tissue is skin *in vitro* or *in vivo*.

50. A method according to any one of claims 47 to 49, in which mitogenesis is promoted by administering an effective amount of the polypeptide to a subject in need of such treatment.

51. A method according to claim 50, in which wound or tissue repair is stimulated.

52. A method according to claim 50 or claim 51, in which the subject is suffering from a condition selected from the group consisting of cutaneous wounds, burns, corneal wounds, an injury to an epithelial cell-lined hollow organ, chronic ulcers, diabetic ulcers, non-healing (trophic) conditions, and diabetes.

53. A method for detecting the presence of a disease, comprising the steps of

(a) contacting a sample from a subject suspected to be suffering from the disease with an antibody according to any one of claims 30 or claim 32 under suitable conditions so as to form a complex between the antibody and an epitope present in the protein; and

(c) comparing the quantitatively determined amount of

complex with the amount present in a sample from a normal subject;

in which the presence of a significantly different amount indicates the presence of a disease.

54. A method according to claim 53, in which the complex is detected by an ELISA assay.

55. A method according to claim 53, in which the antibody is labelled with a radioactive isotope.

56. A method for detecting the presence of a disease, comprising the steps of

(a) quantitatively determining the amount of a BTC-β polypeptide according to any one of claims 16 to 23 present in a sample from a subject suspected to be suffering from the disease, and

(b) comparing the amount so determined with the amount present in a sample from a normal subject;

in which the presence of a significantly different amount indicates the presence of a disease.

57. A method for detecting the presence of a disease, comprising the steps of

(a) quantitatively determining the amount of a polynucleotide according to any one of claims 1 to 13 present in a sample from a subject suspected to be suffering from the disease, and

(b) comparing the amount so determined with the amount present in a sample from a normal subject;

in which the presence of a significantly different amount indicates the presence of a disease.

58. A method according to any one of claims 53 to 57, in which the disease is a hyperproliferative disease selected from the group consisting of breast cancer, epidermoid carcinoma, glioblastoma and pancreatic cancer.

59. A method according to claim 58, in which the disease is a pancreatic dysfunction.

60. A method according to claim 59, in which the disease is diabetes.

Figure 1: Detection of BTC-β and BTC in MCF-7 cells and human breast skin fibroblasts

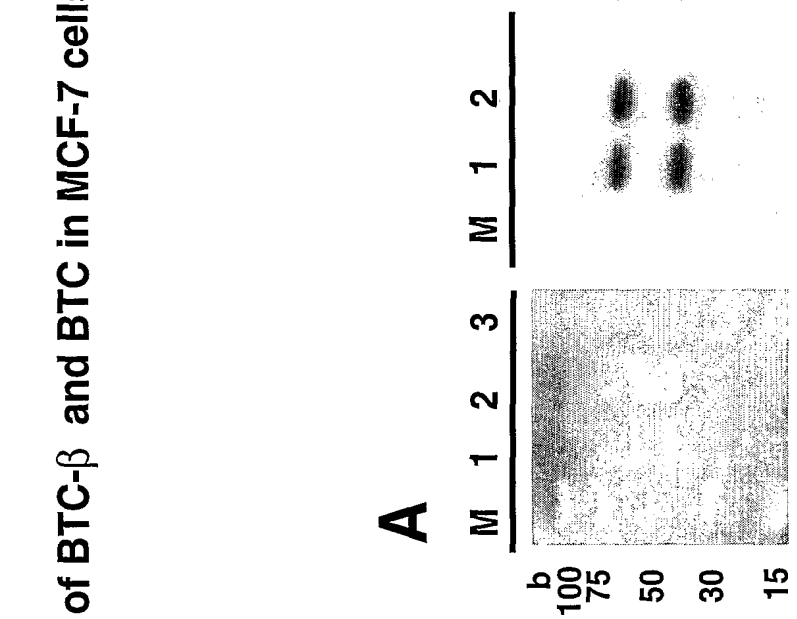
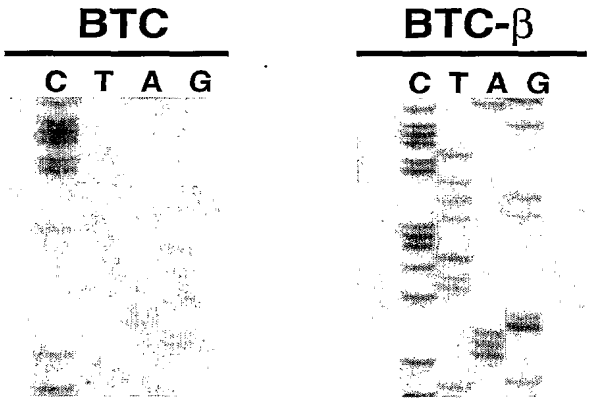


Figure 2: Nucleotide and deduced amino acid sequence of BTC-

A




```
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BTC:    5′ CG CCC TCC TGT GTC TGT GAT GAA GGC TAC 3′

                    P   S   C   V↓  P   L   R   K   R
BTC-β:  5′ CG CCC TCC TGT GTC CCT CTT CGG AAA CGT 3′
```

B

```
                                    M   D   R   A   A   R   C   S   G   A
5′ GCCCGGCCCGGCAGAGCGGGGTTG ATG GAC CGG GCC GCC CGG TGC AGC GGC GCC

 S   S   L   P   L   L   L   A   L   A   L   G   L   V   I   L   H
AGC TCC CTG CCA CTG CTC CTG GCC CTT GCC CTG GGT CTA GTG ATC CTT CAC

 C   V   V   A   D   G   N   S   T   R   S   P   E   T   N   G   L
TGT GTG GTG GCA GAT GGG AAT TCC ACC AGA AGT CCT GAA ACT AAT GGC CTC

 L   C   G   D   P   E   E   N   C   A   A   T   T   T   Q   S   K
CTC TGT GGA GAC CCT GAG GAA AAC TGT GCA GCT ACC ACC ACA CAA TCA AAG

 R   K   G   H   F   S   R   C   P   K   Q   Y   K   H   Y   C   I
CGG AAA GGC CAC TTC TCT AGG TGC CCC AAG CAA TAC AAG CAT TAC TGC ATC

 K   G   R   C   R   F   V   V   A   E   Q   T   P   S   C   V   P
AAA GGG AGA TGC CGC TTC GTG GTG GCC GAG CAG ACG CCC TCC TGT GTC CCT

 L   R   K   R   R   K   R   K   K   K   E   E   E   M   E   T   L
CTT CGG AAA CGT CGT AAA AGA AAG AAG AAA GAA GAA GAA ATG GAA ACT CTG

 G   K   D   I   T   P   I   N   E   D   I   E   E   T   N   I   A
GGT AAA GAT ATA ACT CCT ATC AAT GAA GAT ATT GAA GAG ACA AAT ATT GCT

End
TAA AAGGCTATGAAGTTACCTCCAGGTTGG 3′
```

Figure 3: Comparison of the amino acid sequence and structure of BTC and BTC-

```
BTC:    NH2-MDRAARCSGASSLPLLLALALGLVILHCVVADGNSTRSPETNGLLCGDPEENCAATTTQSKRKGHFSRCPKQYKHYCIKGR 81

BTC-β: NH2-MDRAARCSGASSLPLLLALALGLVILHCVVADGNSTRSPETNGLLCGDPEENCAATTTQSKRKGHFSRCPKQYKHYCIKGR 81

BTC:        CRFVVAEQTPSCVCDEGYIGARCERVDLFYLRGDRGQILVICLIAVMVVFIILVIGVCTCCHPLRKRRKRKKKEEEMETLG 162

BTC-β:      CRFVVAEQTPSCV----------------------------------------------------PLRKRRKRKKKEEEMETLG 113

BTC:        KDITPINEDIEETNIA-COOH2 178

BTC-β:      KDITPINEDIEETNIA-COOH2 129
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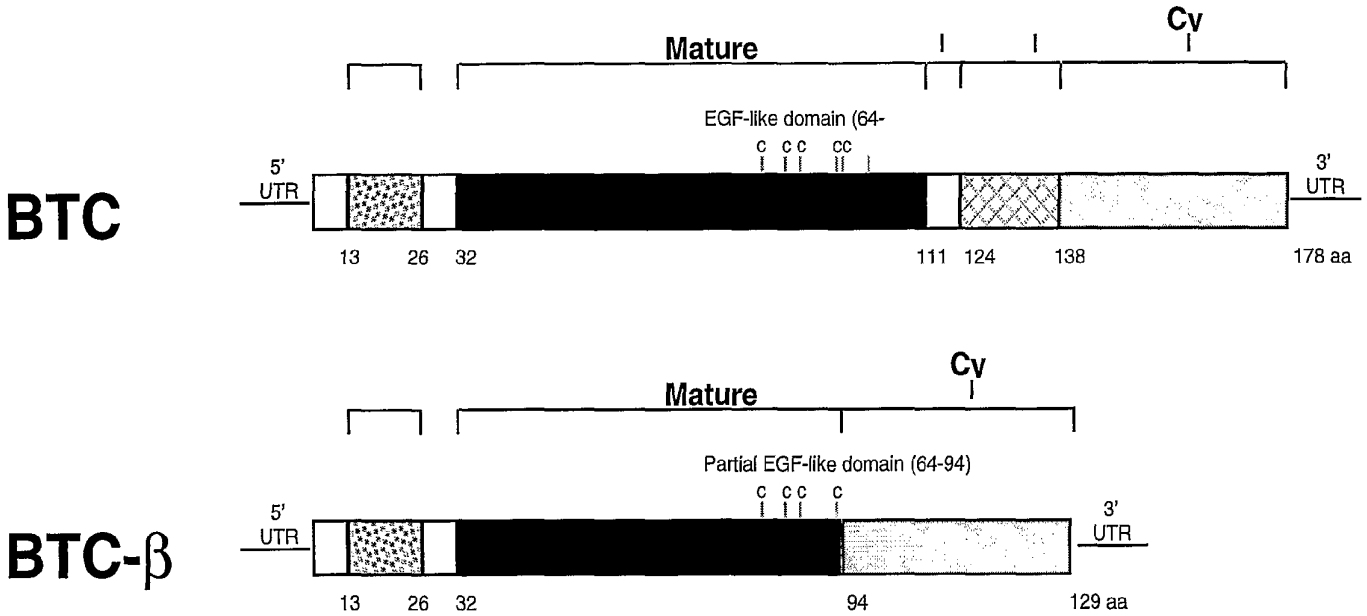

Figure 4: BTC-β polypeptides

BTC-β_{1-129} NH_2-MDRAARCSGASSLPLLLALALGLVILHCVVADGNSTRSPETNGLLCGDPEENCAATTT
QSKRKGHFSRCPKQYKHYCIKGRCRFVVAEQTPSCVPLRKRRKRKKKEEEMETLGKDI
TPINEDIEETNIA-$COOH_2$

BTC-β_{1-94} NH_2-MDRAARCSGASSLPLLLALALGLVILHCVVADGNSTRSPETNGLLCGDPEENCAATTT
QSKRKGHFSRCPKQYKHYCIKGRCRFVVAEQTPSCV-$COOH_2$

BTC-β_{32-94} NH_2-DGNSTRSPETNGLLCGDPEENCAATTTQSKRKGHFSRCPKQYKHYCIKGRCRFVVAEQ
TPSCV-$COOH_2$

BTC-β_{32-111} NH_2-
DGNSTRSPETNGLLCGDPEENCAATTTQSKRKGHFSRCPKQYKHYCIKGRCRFVVAEQ
TPSCVPLRKRRKRKKKEEEMET-$COOH_2$

BTC-β_{32-129} NH_2-
DGNSTRSPETNGLLCGDPEENCAATTTQSKRKGHFSRCPKQYKHYCIKGRCRFVVAEQ
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Figure 5: BTC-β mRNA expression in various human

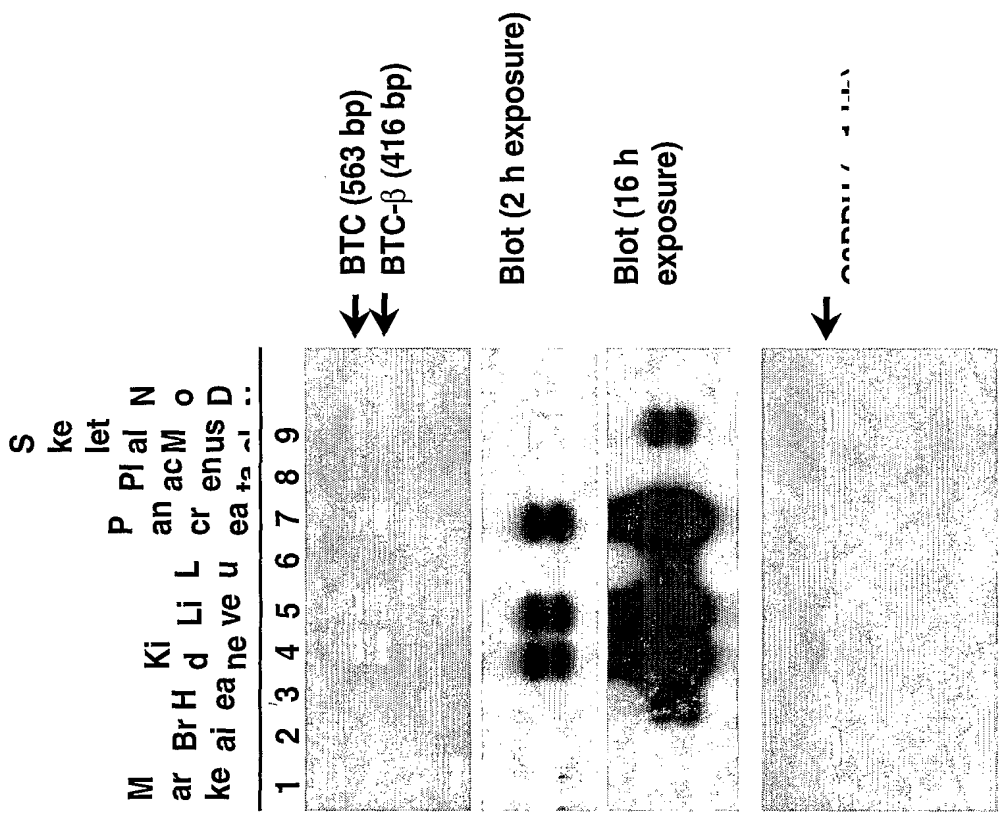
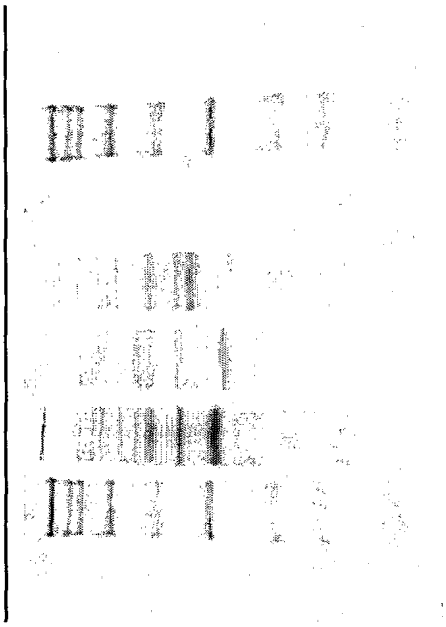


Figure 6: SDS-PAGE analysis of bacterially expressed BTC-β polypeptides.



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35 40 45

Gly Arg Cys Arg Phe Val Val Ala Glu Gln Thr Pro Ser Cys Val

Pro

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU01/00010

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: C12N 15/12, 15/19; C07K 14/71, 16/22; A61K 38/18, 39/395; G01N 33/68; C12Q 1/68.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

CHEMICAL ABSTRACTS (CA) Subsequences and Keywords (KW)- see electronic database box below.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

GENBANK, SWISS PROT, PIR AND EMBL: Subsequences - see electronic database box below.

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Sequences: SEQ ID NOS 1-6 (continued in the supplemental box).

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DUNBAR A *et al* "Identification of betacellulin as a major peptide growth factor in milk: purification, characterization and molecular cloning of bovine betacellulin." *Biochem J* (1999) **344** pp713-721. See page 720 (first column) and figure 8.	30-32, 36 and 53-55.
X	GenBank accession no. K03220. 14 January 1995. See the whole document.	1-6, 9 and 29.
X	GenBank accession no. K03221. 14 January 1995. See the whole document.	1-6, 9 and 29.

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
16 March 2001

Date of mailing of the international search report
21 March 2001

Name and mailing address of the ISA/AU

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU01/00010

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GenPept accession no. AAA61158. 14 January 1995. See the whole document.	16-18, 24-26, 28, 38 and 39.
A	Abe Y *et al* "Disulfide bond structure of human epidermal growth factor receptor." *J Biol Chem* **273** pp11150-11157 (1998).	
A	WO 99/62955 A (BIOMOLECULAR RESEARCH INSTITUTE LIMITED *et al*) 9 December 1999.	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU01/00010

Box I **Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos :

because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos : **17 and 24 (and their appending claims)**

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

- See supplemental box.

3. ☐ Claims Nos :

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II **Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Supplemental Box
(To be used when the space in any of Boxes I to VIII is not sufficient)

Continuation of Box No 1.2:

Claim 17 defines a splice variant of human BTC, fragment or analogues thereof, with the ability to bind an ErbB receptor; however, there is no structural definition of the variant in the claim. Claim 24 defines an analogous growth factor variant of the EBG family of receptors in which one or more of the amino acid residues normally present are absent. The ambit of these claims so defined is broad, as such it would not be economical to fully search their scopes. Further the invention as disclosed in the specification is to the BTC variant in which the C_5-C_6 disulphide loop normally present in ERB receptors is absent. Clearly the scope of claims 17 and 24 is not restricted to such an inventive concept.

Continuation of box marked "Electronic data base consulted during the international search" in **FIELDS SEARCHED:**
Subsequences (a) C.{7}C.{4}C.{10}C.[-C].{8}C; (b) C.{7}C.{4}C.{10}C.C.{8}[-C];
(c) C.{7}C.{4}C.{10}C.[-C].{8}[-C]; (d) C.{7}C.{4}C.{10}C.{1,10}[-C]^; and (e) C.{7}C.{4}C.{10}C.{1,10}^;
any of the above subsequences and epidermal growth factor.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/AU01/00010

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member			
WO	9962955	AU	40245/99	EP	1082345

END OF ANNEX